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54 **Monoclonal antibody.**

57 **Monoclonal antibodies exhibiting specific reactivities to human TNF- α are disclosed. The antibodies are useful as a medicine and ensure immunological purification and measurement of said human TNF.**

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not human 76

MONOCLONAL ANTIBODY

BACKGROUND OF THE INVENTION5 Field of the Invention:

The present invention relates to monoclonal antibodies for human TNFs (tumor necrosis factors), and, more particularly, to monoclonal antibodies for a human TNF which are useful as a medicine and ensure immunological purification and measurement of said human TNF.

10

Description of the Background Art:

A lipopolysaccharide (LPS), when administered to a mouse, rabbit, or rat sensitized by *Bacillus de*
 15 *Calmette Guerin* (BCG), induces a factor hemorrhagically necrosing tumors in serum. *Carswell et al.* named this factor "TNF" in 1975 [*Proc. Nat. Acad. Sci., USA*, **72**, 3666 (1975)]. Macrophages proliferate in the spleen of a mouse sensitized with BCG but elapse by the administration of LPS. From this, TNF has been considered to be produced by macrophages. Recent studies have unveiled the fact that an *in vitro* LPS treatment of isolated macrophages induced a TNF activity in the supernatant of the macrophage
 20 culture broth, confirming that macrophages are TNF-producing cells. Several leukemia cells producing TNF have also been reported.

The characteristic of TNF destroying various cancer cells and inhibiting their growth, while exhibiting no such effects on normal cells, suggests its potential utility as a carcinostatic agent. The anti-tumor activity of TNF has been confirmed in experiments using a purified TNF derived from mice and rabbits.

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The molecular weights of TNF produced by various animals as determined by a gel filtration analysis are reported to be 150,000 and 40,000-60,000 for TNF produced by mouse, 67,000 and 39,000 for TNF
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In 1984, the cloning of TNF-cDNA was successfully achieved using human myelomà leukemia cells "HL-60" which are a type of TNF-producing cell. This has ensured large-scale production of TNF using *E.*
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The amino acid sequence of TNF and the gene base sequence have respectively 28% and 46%
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As mentioned above, because of its special physiological activity, the utility of TNF as a medicine can
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SUMMARY OF THE INVENTION

Accordingly, an object of the present invention is to provide monoclonal antibodies exhibiting specific reactivities to human TNF- α which are useful as a medicine and ensure immunological purification and measurement of said human TNF.

Other objects, features and advantages of the invention will hereinafter become more readily apparent from the following description.

DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS

According to the present invention monoclonal antibodies having a specificity for human TNF- α is provided.

A novel and simple immunoassay technique for measuring said human TNF- α with high sensitivity and with high precision can be provided by the use of the monoclonal antibodies of the present invention.

Since the monoclonal antibodies of the present invention are specific with respect to TNF- α , they can be utilized for a specific purification of TNF- α if applied to affinity chromatography or the like.

The antibodies of the present invention are hereinafter described in detail.

The antibodies of the present invention can be prepared by using a TNF- α as an immune antigen. More specifically, a hybridoma is first prepared from mammal phagocytes (immunocytes) immunized with said immune antigen and mammal plasmacytoma cells. A clone capable of producing a desired antibody is selected from the hybridoma, cultured, and collected.

There are no specific limitations as to the types of TNF- α used as an immune antigen in this method. A supernatant of a culture medium containing a human TNF induced by the application of a known *in vitro* method or its purified product [*Proc. Natl. Acad. Sci., USA*, **82**, 6637 (1985)], a human TNF produced by the use of a gene recombinant technique [*Nature*, **312**, 724-729 (1984)], or a synthetic peptide prepared having a part of the amino acid sequence of one of these TNFs can be used.

There are no specific limitations as to mammals to be immunized with the immune antigen. It is desirable, however, that the mammal be selected taking into consideration its compatibility with plasmacytoma cells used for the cell fusion. Usually, the use of mouse or rat is advantageous.

Immunization can be performed by administering said immune antigen to a mammal by intravenous, subcutaneous, intradermic, or intraperitoneal injection. More specifically, the immune antigen, if desired, mixed with a conventional adjuvant, is administered to an animal, e.g. mouse, once a day at 3-5 intervals until the total amount administered becomes about 100-500 μ g/mouse. Preferable immune antigens are spleen cells extracted about 3 days after the above final administration.

There are several cells known in the art as plasmacytoma cells of mammals which can be used as the parent cells to be fused with the immune cells. They are, for example, myeloma tumor cells such as p3 (p3/x63-Ag8) [*Nature*, **256**, 495-497 (1975)], p3-U1 [*Current Topics in Microbiology and Immunology*, **81**, 1-7 (1978)], NS-1 [*Eur. J. Immunol.*, **6**, 511-519 (1976)], MPC-11 [*Cell*, **8**, 405-415 (1976)], SP2/O [*Nature*, **276**, 269-270 (1978)], FO [*J. Immunol. Meth.*, **35**, 1-21 (1980)], x63.6.5.3. [*J. Immunol.*, **123**, 1548-1550 (1979)], S194 [*J. Exp. Med.*, **148**, 313-323 (1978)], etc., rat R210 [*Nature*, **277**, 131-133 (1979)], and the like.

Fusion of these plasmacytoma cells and the above immune cells can be carried out according to a known method, for example, according to the method of Milstein [*Method in Enzymology*, **73**, 3 (1981)]. Specifically, the cell fusion can be carried out in a normal culture medium in the presence of a conventional cell fusion promoter such as polyethylene glycol (PEG), Sendai virus (HVJ), etc. An auxiliary agent such as dimethylsulfoxide may be added to the culture medium, if necessary for further promoting the cell fusion efficiency. The ratio of plasmacytoma cells and immune cells used may be a ratio commonly employed in the art. Usually, immune cells are used in an amount of 1-10 times plasmacytoma cells. Various media commonly used for culturing plasmacytoma cells, RPMI-1640 medium, MEM medium, etc., can be used for the cell fusion. It is desirable that serum replenishers such as fetus cattle serum (FCS) be eliminated from the medium. The cell fusion is carried out by thoroughly mixing a prescribed amount of plasmacytoma cells and immune cells in a medium and by adding to it about 30-60 w/v% of a PEG solution having an average molecular weight of 1,000-6,000 which is heated in advance to about 37°C. Thereafter, the addition of a suitable medium and centrifugation to remove the supernatant are repeated until a desired hybridoma is formed.

Separation of the hybridoma can be carried out by culturing the hybridoma in a conventional selection medium, such as, for example, HAT medium (a medium containing hypoxanthine, aminopterin, and thymidine). The culture is carried out for a period of time, usually several days to several weeks, sufficient to destroy cells other than target hybridoma such as unfused cells and the like. The hybridoma thus produced is subjected to screening of the target antibody by means of a conventional limiting dilution analysis and to monocloning.

The screening of the target antibody can be carried out by any one of various methods commonly used for antibody screening, such as the ELISA method [Engvall, E., *Meth. Enzymol.*, **70**, 419-439 (1980)], the plaque method, the spot method, the agglutination reaction method, the Ouchterlony method, the radio-immunoassay (RIA) method, and the like ["Hybridoma and Monoclonal Antibody", R&D Planning Publishing Co., 30-53 (1982)]. The above-mentioned immune antigen can be used for the screening.

The hybridoma thus obtained capable of producing desired monoclonal antibodies which can identify human TNF- α can be subcultured in a normal medium and can be stored in liquid nitrogen for a long period of time.

Collection of desired antibodies from the hybridoma can be performed, for example, by culturing the hybridoma according to a conventional method and collecting the supernatant containing antibodies, or by administering the hybridoma to a mammal having compatibility therewith, allowing the hybridoma to grow, and collecting the antibodies from the abdominal dropsy. The former method is suitable for preparing a high-purity antibody and the latter can be applied to a large-scale production of antibodies.

The antibodies thus produced can be purified by a conventional method such as salting-out, gel permeation, affinity chromatography, or the like.

The monoclonal antibodies of the present invention thus prepared have a specificity for TNF- α .

Among the antibodies of the present invention, there is one of a type having a neutralizing effect on human TNF- α biological activity. This type of antibody is particularly suitable for the specific measurement of human TNF- α having a biological activity. Also, among the antibodies of the present invention, there is one of a type which is capable of recognizing specifically different sites of TNF- α , exhibits no steric hindrance between themselves, and, at the same time, can combine with TNF- α . Such an antibody is effective as a sample for the sandwich technique and the like. In addition, there is another type of antibody among the antibodies of the present invention which exhibits a particularly superior reactivity in liquid-phase or solid-phase systems. This type of antibody is suitable for use in liquid- or solid-phase immunoassay.

According to the present invention monoclonal antibodies which have a specificity to human TNF- α are provided. Also, an immunoassay technique having extremely high sensitivity and superior specificity can be provided by the use of antibodies of the present invention. The immunoassay technique can detect and measure with a very high precision human TNF- α in samples such as clinical samples containing even an extremely small amount of human TNF- α .

Other features of the invention will become apparent in the course of the following description of the exemplary embodiments which are given for illustration of the invention and are not intended to be limiting thereof.

EXAMPLES

Example 1

Preparation of Antibodies of the Present Invention

A human TNF- α (10-20 μ g) prepared by a gene recombinant technique [*Nature*, **312**, 724-729 (1984)] was intraperitoneally administered to a BALB/c mouse together with a complete Freund's adjuvant. The same amount of the human TNF- α was additionally administered twice together with an incomplete Freund's adjuvant, first 3-4 weeks after the initial administration and second 3-4 weeks thereafter, thus immunizing the mouse. After 3-4 days following the last administration, cell fusion was performed according to a conventional method [*Method in Enzymology*, **73**, 3 (1981)], using immunized spleen cells and myeloma tumor cells [P3U1, *Current Topics in Microbiology*, **81**, 1-7 (1978)] at a ratio of 10:1 in polyethylene glycol (PEG-4000). The hybridoma was screened with the HAT medium. The supernatant was tested by an enzymatic immunoassay technique using a 96-well microplate coated with said human TNF- α .

and a (goat) peroxidase-labeled anti-mouse IgG (product of E. Y. Laboratories, Inc.), thereby detecting cells capable of producing the target antibody for the human TNF- α .

Cloning by a limiting dilution technique was repeated to produce 7 strains of clone capable of producing the desired antibodies. They were named KOCO701, KOCO702, KOCO703, KOCO704, KOCO705, KOCO706, and KOCO707. The antibodies produced by them were respectively named ANOC701, ANOC702, ANOC703, ANOC704, ANOC705, ANOC706, and ANOC707.

One of the strains, the hybridoma producing an antibody with the present invention, "KOCO 705", was deposited to the Fermentation Research Institute under the name of KOCO 705 (FERM BP-2569).

Characteristics of the antibodies produced by each of the above clones are as follows:

(1) Antibody Subclass

The subclasses were determined by using a mouse antibody subclass detection kit (product of Bio-Rad Co.). The results are given in Table 1.

TABLE 1

| Antibody No. | Subclass |
|--------------|-------------------|
| ANOC701 | IgG ₁ |
| ANOC702 | IgG ₁ |
| ANOC703 | IgG _{2a} |
| ANOC704 | IgG ₁ |
| ANOC705 | IgG ₁ |
| ANOC706 | IgG ₁ |
| ANOC707 | IgG ₁ |

(2) Antibody Production Level

IgG in ascites was purified using the Protein A affinity, and the concentration (mg/ml) was determined by OD₂₈₀ adsorption. The OD₂₈₀ value at a 1 mg/ml IgG concentration was assumed to be 1.4.

The results are shown in Table 2.

TABLE 2

| Antibody No. | IgG (mg/ml) |
|--------------|-------------|
| ANOC701 | 0.1 |
| ANOC702 | 1.6 |
| ANOC703 | 0.2 |
| ANOC704 | 1.2 |
| ANOC705 | 1.1 |
| ANOC706 | 8.6 |
| ANOC707 | 2.0 |

(3) Neutralization Antibody Titer

The neutralization antibody titer of the ascites was determined by the LM assay technique which is a type of TNF bioassay. The neutralization antibody titer is defined as the unit numbers of a TNF- α which can

be neutralized by 1 ml of an ascites to one unit.
The results are shown in Table 3.

TABLE 3

| Antibody No. | Neutralization Antibody Titer (U) |
|--------------|--------------------------------------|
| ANOC701 | 32,000 < |
| ANOC702 | 32,000 < |
| ANOC703 | 2,000 > |
| ANOC704 | 32,000 < |
| ANOC705 | 32,000 < |
| ANOC706 | 32,000 < |
| ANOC707 | 32,000 < |

(4) Molecular Weight

Hybridoma cultured in the abdomen of a mouse was purified into IgG₁ using an IgG purification kit (MOPS Kit, product of Bio-rad Co.). The IgG's heavy-chain and light-chain molecular weights were determined by electrophoresis using SDS-PAGE in the presence of 2ME. The sum of the two molecular weights was taken as the monoclonal antibodies.

The results are shown in Table 4.

TABLE 4

| Antibody No. | Molecular Weight | | |
|--------------|------------------|----------------|------|
| | Heavy chain | Light chain | IgG |
| ANOC701 | N.T. | N.T. | N.T. |
| ANOC702 | 53.0 | 25.0 | 156 |
| ANOC703 | 53.0 | 24.5 | 155 |
| ANOC704 | 52.0 | 24.0 | 152 |
| ANOC705 | 53.0 | 24.5 | 155 |
| ANOC706 | 53.0 | 23.0 | 152 |
| ANOC707 | 50.0 | 24.0 | 148 |

(5) Western Blotting Analysis

Specificity for TNF- α and non-reactivity with *E. coli* of the antibodies of the present invention were confirmed in the following manner. γ TNF- α and γ TNF- α -producing *E. coli* were treated with an SDS lysis buffer to a final concentration of 100 μ g/ml. SDS-PAGE was performed to effect blotting onto nitrocellulose and to determine the reactivity of each monoclonal antibody.

The results are shown in Table 5.

TABLE 5

| Antibody No. | Reactivity | |
|--------------|---------------------------|---------------|
| | <i>E. coli</i> protein | TNF- α |
| ANOC701 | - | + |
| ANOC702 | - | + |
| ANOC703 | - | - |
| ANOC704 | - | + |
| ANOC705 | - | + |
| ANOC706 | - | + |
| ANOC707 | - | + |

Table 5 shows that ANOC701, ANOC702, ANOC704, ANOC705, ANOC706, and ANOC707 combine with TNF- α with specificity and do not react with *E. coli* protein. The non-reactivity of ANOC703 with γ TNF- α suggests its recognition of a steric structure.

(6) ELISA Sensitivity

Each 100 μ l of monoclonal antibodies ANOC701-ANOC707 diluted with PBS to a concentration of 10 μ g/ml was poured into each well of a 96-well microplate and left overnight. Holes were blocked with 1% skim milk. 100 μ l of a standard TNF- α was added to each well and incubated at 4°C overnight. After washing the plate, 100 μ l of a 1,000-fold solution of a rabbit polyclonal antibody (OCT701) against TNF- α was added and incubated for 2 hours at room temperature. The plate was washed and 100 μ l of (goat) POD-labeled anti-rabbit IgG was added to each well. After incubation for 2 hours at room temperature, the plate was washed and the enzymatic activity of POD was measured.

The ELISA sensitivity of a monoclonal antibody is expressed by the TNF- α concentration of the antibody, the difference of which OD₄₉₂ adsorption and the OD₄₉₂ adsorption without TNF- α is 0.1.

The results are shown in Table 6.

TABLE 6

| Antibody No. | ELISA Sensitivity (OD ₄₉₂ = 0.1) |
|--------------|--|
| ANOC701 | N.T. |
| ANOC702 | 1.0 ng/ml (0.10 ng/well) |
| ANOC703 | N.T. |
| ANOC704 | 1.2 ng/ml (0.12 ng/well) |
| ANOC705 | 0.44 ng/ml (0.044 ng/well) |
| ANOC706 | 0.86 ng/ml (0.086 ng/well) |
| ANOC707 | 1.0 ng/ml (0.10 ng/well) |

(8) Standard Curve by ELISA Method

100 μ l of monoclonal antibody ANOC705 diluted with PBS to a concentration of 10 μ g/ml was poured into each well of a 96-well microplate and left overnight at 4°C. Wells were blocked with 1% skim milk. 100 μ l of a standard TNF- α was added in triplicate to each well and incubated at 4°C overnight. After washing the plate, 100 μ l of a 1,000-fold solution of a rabbit polyclonal antibody (OCT701) against TNF- α was added and incubated for 2 hours at room temperature. The plate was washed and 100 μ l of (goat) POD-labeled anti-rabbit IgG was added to each well. After incubation for 2 hours at room temperature, the plate was

washed and the enzymatic activity as OD₄₉₂ adsorbance was measured using o-phenylenedimine as a substrate.

The results are shown in Table 7.

TABLE 7

| TNF- α concentration (pg/ml) | 0 | 9.375 | 37.50 | 150.0 | 600.0 |
|-------------------------------------|-----|-------|-------|-------|-------|
| Adsorbance 1 | 89 | 117 | 205 | 535 | 1510 |
| Adsorbance 2 | 95 | 123 | 206 | 540 | 1481 |
| Adsorbance 3 | 95 | 124 | 209 | 514 | 1487 |
| Mean | 93 | 121 | 207 | 530 | 1490 |
| Mean - Blank | 0 | 28 | 114 | 437 | 1397 |
| C. V. (%) | 3.7 | 3.1 | 1.0 | 2.6 | 0.7 |

Obviously, numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

Claims

1. Monoclonal antibodies exhibiting specific reactivities to human TNF- α .



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number

EP 89 11 9648

| DOCUMENTS CONSIDERED TO BE RELEVANT | | | |
|---|---|--|--|
| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | CLASSIFICATION OF THE APPLICATION (Int. Cl.5) |
| X | Hybridoma, Vol. 6, No. 4, p. 359-370, 1987 B.M. Fendly et al., "Murine monoclonal Antibodies defining neutralizing epitopes on Tumor Necrosis Factor" * The entire article * | 1 | C 12 P 21/08 // A 61 K 39/395 G 01 N 33/577 G 01 N 33/68 C 07 K 3/18 |
| X | Hybridoma, Vol. 6, No 5, p. 489-507, 1987 T.S. Bringman et al., "Monoclonal antibodies to human Tumor Necrosis Factors alpha and beta: Application for affinity purification, immunoassays, and as structural probes" * The entire article * | 1 | |
| | | | TECHNICAL FIELDS SEARCHED (Int. Cl.5) |
| | | | C 12 P |
| The present search report has been drawn up for all claims | | | |
| Place of search THE HAGUE | | Date of completion of the search 16-01-1990 | Examiner RYCKEBOSCH A.O.A. |
| CATEGORY OF CITED DOCUMENTS | | | |
| X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document | | T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document | |

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EUROPEAN PATENT SPECIFICATION

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There are no specific limitations as to the types of TNF- α used as an immune antigen in this method. A supernatant of a culture medium containing a human TNF induced by the application of a known *in vitro* method or its purified product [*Proc. Natl. Acad. Sci., USA*, **82**, 6637 (1985)], a human TNF produced by the use of a gene recombinant technique [*Nature*, **312**, 724-729 (1984)], or a synthetic peptide prepared having a part of the amino acid sequence of one of these TNFs can be used.

There are no specific limitations as to mammals to be immunized with the immune antigen. It is desirable, however, that the mammal be selected taking into consideration its compatibility with plasmacytoma cells used for the cell fusion. Usually, the use of mouse or rat is advantageous.

Immunization can be performed by administering said immune antigen to a mammal by intravenous, subcutaneous, intradermic, or intraperitoneal injection. More specifically, the immune antigen, if desired, mixed with a conventional adjuvant, is administered to an animal, e.g. mouse, once a day at 3-5 intervals until the total amount administered becomes about 100-500 μ g/mouse. Preferable immune antigens are spleen cells extracted about 3 days after the above final administration.

There are several cells known in the art as plasmacytoma cells of mammals which can be used as the parent cells to be fused with the immune cells. They are, for example, myeloma tumor cells such as p3 (p3/x63-Ag8) [*Nature*, **256**, 495-497 (1975)], p3-U1 [*Current Topics in Microbiology and Immunology*, **81**, 1-7 (1978)], NS-1 [*Eur. J. Immunol.*, **6**, 511-519 (1976)], MPC-11 [*Cell*, **8**, 405-415 (1976)], SP2/O [*Nature*, **276**, 269-270 (1978)], FO [*J. Immunol. Meth.*, **35**, 1-21 (1980)], x63.6.5.3. [*J. Immunol.*, **123**, 1548-1550 (1979)], S194 [*J. Exp. Med.*, **148**, 313-323 (1978)], etc., rat R210 [*Nature*, **277**, 131-133 (1979)], and the like.

Fusion of these plasmacytoma cells and the above immune cells can be carried out according to a known method, for example, according to the method of Milstein [*Method in Enzymology*, **73**, 3 (1981)]. Specifically, the cell fusion can be carried out in a normal culture medium in the presence of a conventional cell fusion promoter such as polyethylene glycol (PEG), Sendai virus (HVJ), etc. An auxiliary agent such as dimethylsulfoxide may be added to the culture medium, if necessary for further promoting the cell fusion efficiency. The ratio of plasmacytoma cells and immune cells used may be a ratio commonly employed in the art. Usually, immune cells are used in an amount of 1-10 times plasmacytoma cells. Various media commonly used for culturing plasmacytoma cells, RPMI-1640 medium, MEM medium, etc., can be used for the cell fusion. It is desirable that serum replenishers such as fetus cattle serum (FCS) be eliminated from the medium. The cell fusion is carried out by thoroughly mixing a prescribed amount of plasmacytoma cells and immune cells in a medium and by adding to it about 30-60 w/v% of a PEG solution having an average molecular weight of 1,000-6,000 which is heated in advance to about 37°C. Thereafter, the addition of a suitable medium and centrifugation to remove the supernatant are repeated until a desired hybridoma is formed.

Separation of the hybridoma can be carried out by culturing the hybridoma in a conventional selection medium, such as, for example, HAT medium (a medium containing hypoxanthine, aminopterin, and thymidine). The culture is carried out for a period of time, usually several days to several weeks, sufficient to destroy cells other than target hybridoma such as unfused cells and the like. The hybridoma thus produced is subjected to screening of the target antibody by means of a conventional limiting dilution analysis and to monocloning.

The screening of the target antibody can be carried out by any one of various methods commonly used for antibody screening, such as the ELISA method [Engvall, E., *Meth. Enzymol.*, **70**, 419-439 (1980)], the

plaque method, the spot method, the agglutination reaction method, the Ouchterlony method, the radio-immunoassay (RIA) method, and the like ["Hybridoma and Monoclonal Antibody", R&D Planning Publishing Co., 30-53 (1982)]. The above-mentioned immune antigen can be used for the screening.

The hybridoma thus obtained capable of producing desired monoclonal antibodies which can identify human TNF- α can be subcultured in a normal medium and can be stored in liquid nitrogen for a long period of time.

Collection of desired antibodies from the hybridoma can be performed, for example, by culturing the hybridoma according to a conventional method and collecting the supernatant containing antibodies, or by administering the hybridoma to a mammal having compatibility therewith, allowing the hybridoma to grow, and collecting the antibodies from the abdominal dropsy. The former method is suitable for preparing a high-purity antibody and the latter can be applied to a large-scale production of antibodies.

The antibodies thus produced can be purified by a conventional method such as salting-out, gel permeation, affinity chromatography, or the like.

The monoclonal antibodies of the present invention thus prepared have a specificity for TNF- α .

The antibodies of the present invention have a neutralizing effect on human TNF- α biological activity. This antibody is particularly suitable for the specific measurement of human TNF- α having a biological activity. Also, among the antibodies, described here, there is one of a type which is capable of recognizing specifically different sites of TNF- α , exhibits no steric hindrance between themselves, and, at the same time, can combine with TNF- α . Such an antibody is effective as a sample for the sandwich technique and the like. In addition, the antibodies of the present invention exhibit a particularly superior reactivity in liquid-phase or solid-phase systems. This antibody is suitable for use in liquid- or solid-phase immunoassay.

According to the present invention monoclonal antibodies which have a specificity to human TNF- α are provided. Also, an immunoassay technique having extremely high sensitivity and superior specificity can be provided by the use of antibodies of the present invention. The immunoassay technique can detect and measure with a very high precision human TNF- α in samples such as clinical samples containing even an extremely small amount of human TNF- α .

Other features of the invention will become apparent in the course of the following description of the exemplary embodiments which are given for illustration of the invention and are not intended to be limiting thereof.

EXAMPLES

Example 1

Preparation of Antibodies of the Present Invention

A human TNF- α (10-20 μ g) prepared by a gene recombinant technique [*Nature*, 312, 724-729 (1984)] was intraperitoneally administered to a BALB/c mouse together with a complete Freund's adjuvant. The same amount of the human TNF- α was additionally administered twice together with an incomplete Freund's adjuvant, first 3-4 weeks after the initial administration and second 3-4 weeks thereafter, thus immunizing the mouse. After 3-4 days following the last administration, cell fusion was performed according to a conventional method [*Method in Enzymology*, 73, 3 (1981)], using immunized spleen cells and myeloma tumor cells [P3U1, *Current Topics in Microbiology*, 81, 1-7 (1978)] at a ratio of 10:1 in polyethylene glycol (PEG-4000). The hybridoma was screened with the HAT medium. The supernatant was tested by an enzymatic immunoassay technique using a 96-well microplate coated with said human TNF- α and a (goat) peroxidase-labeled anti-mouse IgG (product of E. Y. Laboratories, Inc.), thereby detecting cells capable of producing the target antibody for the human TNF- α .

Cloning by a limiting dilution technique was repeated to produce 7 strains of clone capable of producing the desired antibodies. They were named KOCO701, KOCO702, KOCO703, KOCO704, KOCO705, KOCO706, and KOCO707. The antibodies produced by them were respectively named ANOC701, ANOC702, ANOC703, ANOC704, ANOC705, ANOC706, and ANOC707.

One of the strains, the hybridoma producing an antibody with the present invention, "KOCO 705", was deposited to the Fermentation Research Institute under the name of KOCO 705 (FERM BP-2569).

Characteristics of the antibodies produced by each of the above clones are as follows:

(1) Antibody Subclass

The subclasses were determined by using a mouse antibody subclass detection kit (product of Bio-Rad Co.). The results are given in Table 1.

TABLE 1

| Antibody No. | Subclass |
|--------------|-------------------|
| ANOC701 | IgG ₁ |
| ANOC702 | IgG ₁ |
| ANOC703 | IgG _{2a} |
| ANOC704 | IgG ₁ |
| ANOC705 | IgG ₁ |
| ANOC706 | IgG ₁ |
| ANOC707 | IgG ₁ |

(2) Antibody Production Level

IgG in ascites was purified using the Protein A affinity, and the concentration (mg/ml) was determined by OD₂₈₀ adsorption. The OD₂₈₀ value at a 1 mg/ml IgG concentration was assumed to be 1.4.

The results are shown in Table 2.

TABLE 2

| Antibody No. | IgG (mg/ml) |
|--------------|-------------|
| ANOC701 | 0.1 |
| ANOC702 | 1.6 |
| ANOC703 | 0.2 |
| ANOC704 | 1.2 |
| ANOC705 | 1.1 |
| ANOC706 | 8.6 |
| ANOC707 | 2.0 |

(3) Neutralization Antibody Titer

The neutralization antibody titer of the ascites was determined by the LM assay technique which is a type of TNF bioassay. The neutralization antibody titer is defined as the unit numbers of a TNF- α which can be neutralized by 1 ml of an ascites to one unit.

The results are shown in Table 3.

TABLE 3

| Antibody No. | Neutralization Antibody Titer (U) |
|--------------|-----------------------------------|
| ANOC701 | 32,000 < |
| ANOC702 | 32,000 < |
| ANOC703 | 2,000 > |
| ANOC704 | 32,000 < |
| ANOC705 | 32,000 < |
| ANOC706 | 32,000 < |
| ANOC707 | 32,000 < |

(4) Molecular Weight

Hybridoma cultured in the abdomen of a mouse was purified into IgG₁ using an IgG purification kit (MOPS Kit, product of Bio-rad Co.). The IgG's heavy-chain and light-chain molecular weights were determined by electrophoresis using SDS-PAGE in the presence of 2ME. The sum of the two molecular weights was taken as the monoclonal antibodies.

The results are shown in Table 4.

TABLE 4

| Antibody No. | Molecular Weight | | |
|--------------|------------------|-------------|------|
| | Heavy chain | Light chain | IgG |
| ANOC701 | N.T. | N.T. | N.T. |
| ANOC702 | 53.0 | 25.0 | 156 |
| ANOC703 | 53.0 | 24.5 | 155 |
| ANOC704 | 52.0 | 24.0 | 152 |
| ANOC705 | 53.0 | 24.5 | 155 |
| ANOC706 | 53.0 | 23.0 | 152 |
| ANOC707 | 50.0 | 24.0 | 148 |

(5) Western Blotting Analysis

Specificity for TNF- α and non-reactivity with *E. coli* of the antibodies of the present invention were confirmed in the following manner. γ TNF- α and γ TNF- α -producing *E. coli* were treated with an SDS lysis buffer to a final concentration of 100 μ g/ml. SDS-PAGE was performed to effect blotting onto nitrocellulose and to determine the reactivity of each monoclonal antibody.

The results are shown in Table 5.

TABLE 5

| Antibody No. | Reactivity | |
|--------------|------------------------|---------------|
| | <i>E. coli</i> protein | TNF- α |
| ANOC701 | - | + |
| ANOC702 | - | + |
| ANOC703 | - | - |
| ANOC704 | - | + |
| ANOC705 | - | + |
| ANOC706 | - | + |
| ANOC707 | - | + |

Table 5 shows that ANOC701, ANOC702, ANOC704, ANOC705, ANOC706, and ANOC707 combine with TNF- α with specificity and do not react with *E. coli* protein. The non-reactivity of ANOC703 with γ TNF- α suggests its recognition of a steric structure.

(6) ELISA Sensitivity

Each 100 μ l of monoclonal antibodies ANOC701-ANOC707 diluted with PBS to a concentration of 10 μ g/ml was poured into each well of a 96-well microplate and left overnight. Holes were blocked with 1% skim milk. 100 μ l of a standard TNF- α was added to each well and incubated at 4°C overnight. After washing the plate, 100 μ l of a 1,000-fold solution of a rabbit polyclonal antibody (OCT701) against TNF- α was added and incubated for 2 hours at room temperature. The plate was washed and 100 μ l of (goat) POD-labeled anti-rabbit IgG was added to each well. After incubation for 2 hours at room temperature, the

plate was washed and the enzymatic activity of POD was measured.

The ELISA sensitivity of a monoclonal antibody is expressed by the $\text{TNF-}\alpha$ concentration of the antibody, the difference of which OD_{492} adsorption and the OD_{492} adsorption without $\text{TNF-}\alpha$ is 0.1.

The results are shown in Table 6.

TABLE 6

| Antibody No. | ELISA Sensitivity ($\text{OD}_{492} = 0.1$) |
|--------------|---|
| ANOC701 | N.T. |
| ANOC702 | 1.0 ng/ml (0.10 ng/well) |
| ANOC703 | N.T. |
| ANOC704 | 1.2 ng/ml (0.12 ng/well) |
| ANOC705 | 0.44 ng/ml (0.044 ng/well) |
| ANOC706 | 0.86 ng/ml (0.086 ng/well) |
| ANOC707 | 1.0 ng/ml (0.10 ng/well) |

(8) Standard Curve by ELISA Method

100 μl of monoclonal antibody ANOC705 diluted with PBS to a concentration of 10 $\mu\text{g/ml}$ was poured into each well of a 96-well microplate and left overnight at 4°C. Wells were blocked with 1% skim milk. 100 μl of a standard $\text{TNF-}\alpha$ was added in triplicate to each well and incubated at 4°C overnight. After washing the plate, 100 μl of a 1,000-fold solution of a rabbit polyclonal antibody (OCT701) against $\text{TNF-}\alpha$ was added and incubated for 2 hours at room temperature. The plate was washed and 100 μl of (goat) POD-labeled anti-rabbit IgG was added to each well. After incubation for 2 hours at room temperature, the plate was washed and the enzymatic activity as OD_{492} adsorbance was measured using o-phenylenedimide as a substrate.

The results are shown in Table 7.

TABLE 7

| $\text{TNF-}\alpha$ concentration (pg/ml) | 0 | 9.375 | 37.50 | 150.0 | 600.0 |
|---|-----|-------|-------|-------|-------|
| Adsorbance 1 | 89 | 117 | 205 | 535 | 1510 |
| Adsorbance 2 | 95 | 123 | 206 | 540 | 1481 |
| Adsorbance 3 | 95 | 124 | 209 | 514 | 1487 |
| Mean | 93 | 121 | 207 | 530 | 1490 |
| Mean -Blank | 0 | 28 | 114 | 437 | 1397 |
| C. V. (%) | 3.7 | 3.1 | 1.0 | 2.6 | 0.7 |

Claims

1. Monoclonal antibodies exhibiting specific reactivities to human $\text{TNF-}\alpha$, obtainable from hybridoma KOCO 705 (FERM BP-2569).

Patentansprüche

1. Aus Hybridom KOCO 705 (FERM BP-2569) erhaltliche monoklonale Antikörper, die spezifische Reaktivitäten gegenüber menschlichem $\text{TNF-}\alpha$ zeigen.

Revendications

1. Anticorps monoclonaux présentant des réactivités spécifiques avec $\text{TNF-}\alpha$ humain, pouvant être obtenus à partir de l'hybridome KOCO 705 (FERM BP-2569).